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## Citrate Uptake in Exchange with Intermediates in the Citrate Metabolic Pathway in *Lactococcus lactis* IL1403<sup>∇</sup>

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**Carbohydrate/citrate cometabolism in *Lactococcus lactis* results in the formation of the flavor compound acetoin. Resting cells of strain IL1403(pFL3) rapidly consumed citrate while producing acetoin when substoichiometric concentrations of glucose or L-lactate were present. A proton motive force was generated by electrogenic exchange of citrate and lactate catalyzed by the citrate transporter CitP and proton consumption in decarboxylation reactions in the pathway. In the absence of glucose or L-lactate, citrate consumption was biphasic. During the first phase, hardly any citrate was consumed. In the second phase, citrate was converted rapidly, but without the formation of acetoin. Instead, significant amounts of the intermediates pyruvate and  $\alpha$ -acetolactate, and the end product acetate, were excreted from the cells. It is shown that the intermediates and acetate are excreted in exchange with the uptake of citrate catalyzed by CitP. The availability of exchangeable substrates in the cytoplasm determines both the rate of citrate consumption and the end product profile. It follows that citrate metabolism in *L. lactis* IL1403(pFL3) splits up in two routes after the formation of pyruvate, one the well-characterized route yielding acetoin and the other a new route yielding acetate. The flux distribution between the two branches changes from 85:15 in the presence of L-lactate to 30:70 in the presence of pyruvate. The proton motive force generated was greatest in the presence of L-lactate and zero in the presence of pyruvate, suggesting that the pathway to acetate does not generate proton motive force.**

Citrate fermentation is a strain-specific trait among lactic acid bacteria (LAB) that is associated with the production of carbon dioxide and C<sub>4</sub> flavor compounds (17). During carbohydrate/citrate cometabolism, additional pyruvate from citrate added to the central pyruvate pool in the glycolytic pathway is converted to acetoin (Fig. 1A). Citrate is transported into the cell by the secondary transporter CitP. Inside, citrate is converted to acetate and oxaloacetate catalyzed by citrate lyase. Acetate leaves the cell while oxaloacetate is decarboxylated to pyruvate by a soluble oxaloacetate decarboxylase (36).  $\alpha$ -Acetolactate synthase converts two molecules of pyruvate to one molecule of  $\alpha$ -acetolactate while releasing carbon dioxide. The majority of  $\alpha$ -acetolactate is decarboxylated to acetoin by  $\alpha$ -acetolactate decarboxylase. A small part of the chemically unstable  $\alpha$ -acetolactate results in the formation of diacetyl in a nonenzymatic oxidative decarboxylation reaction (16, 33). Citrate-fermenting LAB strains are valuable for use as starters in the dairy and wine industries since compounds such as carbon dioxide, acetoin, and diacetyl promote the organoleptic properties of fermentation products.

Citrate metabolism in LAB is a metabolic energy-generating pathway. The pathway generates an electrochemical gradient of protons (proton motive force [PMF]) across the cell membrane (5, 27, 28) by a secondary mechanism in which membrane potential ( $\Delta\Psi$ ) and pH gradient are generated in separate steps (21, 22). The transporter CitP catalyzes uptake of

divalent citrate in exchange for monovalent lactate, which results in a membrane potential of physiological polarity (i.e., inside negative) (Fig. 1B). The pH gradient (inside alkaline) is the result of proton consumption in the decarboxylation reactions taking place in the cytoplasm. The pathway functions as an indirect proton pump.

Usually secondary PMF-generating pathways are simple pathways built around a single decarboxylation reaction. A carboxylate substrate is taken up by a transporter and decarboxylated in the cytoplasm, and then the decarboxylation product is excreted by the same transporter in an exchange process. Well-studied examples are malate decarboxylation in *Lactococcus lactis* (malolactic fermentation) (32), oxalate decarboxylation in *Oxalobacter formigenes* (3), and catabolic amino acid decarboxylation pathways (e.g., references 2, 18, 30, 41, and 44). Typically, the transporters in the pathways take up the substrate in exchange with the product of the pathway and are termed precursor/product exchangers (31). Remarkably, in the citrate fermentation pathway in LAB, citrate is taken up in exchange with lactate (citrolactic fermentation), which is not the product of the citrate degradation pathway but the product of glycolysis.

Following the discovery of citrate fermentation as a secondary proton motive force-generating pathway in *Leuconostoc mesenteroides* (27, 28) and subsequent confirmation for *L. lactis* (26), the citrate transporter CitP was extensively characterized by transport studies with membrane vesicles (4, 5, 6, 27). The transporter is a member of the 2-hydroxycarboxylate transporter (2-HCT) family (40) (transporter classification [TC] 2.A.47 [34]), in which the malate/lactate exchanger MleP, which functions in the malolactic fermentation pathway in LAB (32), and the Na<sup>+</sup>-citrate symporter CitS, which functions

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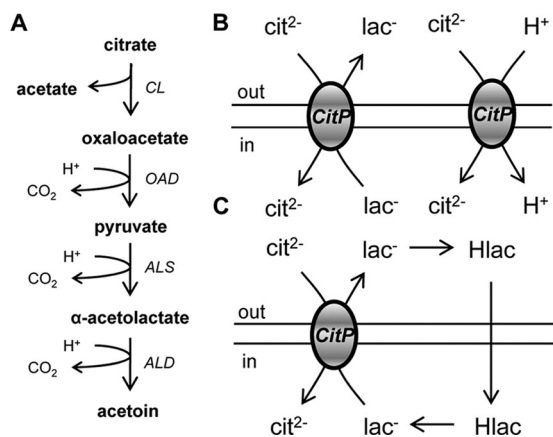


FIG. 1. Citrate metabolism in LAB. (A) Citrate fermentation pathway yielding acetoin. Enzymes: CL, citrate lyase; OAD, oxaloacetate decarboxylase; ALS,  $\alpha$ -acetolactate synthase; ALD,  $\alpha$ -acetolactate decarboxylase. The stoichiometry of the reactions was not taken into account. (B) Kinetic modes of the citrate transporter CitP. Left, exchange (fast) of external citrate ( $\text{cit}^{2-}$ ) and internal L-lactate ( $\text{lac}^-$ ); right, unidirectional uptake (slow) of citrate ( $\text{cit}^{2-}$ ) in symport with a  $\text{H}^+$ . (C) L-Lactate shuttle mechanism. L-Lactate added to the outside of the cells allows CitP to operate in the fast  $\text{cit}^{2-}/\text{lac}^-$  exchange mode by reentering the cell in the permeable protonated state. The net result is the uptake of  $\text{cit}^{2-}$  and a  $\text{H}^+$ .

in citrate fermentation in *Klebsiella pneumoniae* (12), are also found. The name of the family refers to the 2-hydroxycarboxylate motif, i.e.,  $\text{HO-CR}_2\text{-COO}^-$ , shared by the substrates of the transporters in the family (4). Kinetic studies of CitP revealed two modes of transport, symport of divalent citrate with one proton and exchange of divalent citrate with monovalent lactate (Fig. 1B) (25, 27, 35). Since the former was much slower than the latter, it was concluded that CitP is a symporter that was optimized to catalyze exchange, which would be the physiological function. Chase studies with membrane vesicles loaded with radiolabeled citrate showed a remarkable tolerance of CitP (and MleP, but not CitS) to the R substituents of 2-hydroxycarboxylate substrates. CitP accepted R groups differing in size and charge, ranging from H atoms in glycolate to acetyl groups in citrate and everything in between in physiological or nonphysiological substrates. The specificity of CitP for substrates carrying different charges forms the mechanistic basis for membrane potential generation (4, 6).

In this study, the impact of the broad substrate specificity of CitP on the metabolism of citrate in *L. lactis* IL1403 is demonstrated. It is shown that the metabolic intermediates pyruvate and  $\alpha$ -acetolactate and the end product acetate can function in the exchange reaction catalyzed by CitP to take up citrate. The availability of exchangeable metabolites in the cytoplasm determines the end product profile of the pathway. Unexpectedly, following the conversion of citrate to pyruvate, the pathway branched into a route to acetate in addition to the one to acetoin.

#### MATERIALS AND METHODS

**Materials.** M17 broth was obtained from Merck, Darmstadt, Germany. Citrate lyase (catalog no. 10354074001), L-malate dehydrogenase (catalog no. 10127256001), and L-lactate dehydrogenase (catalog no. 10127230001) were obtained from Roche Applied Science. Ethyl 2-acetoxy-2-methylacetoacetate,

$\alpha$ -naphthol, and creatine were obtained from Sigma Aldrich and acetoin, diacetyl, and 2,3-butanediol from Fluka. 3,3'-Dipropylthiocarbocyanine iodide (DiSC<sub>3</sub>) and 2',7'-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein (BCECF; acid form) probes were obtained from Invitrogen Molecular Probes.

**Bacterial strain and growth conditions.** *Lactococcus lactis* IL1403(pFL3), harboring the pFL3 plasmid containing the *citP* gene under the control of the *Streptococcus pneumoniae* *polA* promoter, was used in this study (24). Cells were grown in M17 medium supplemented with 0.5% (wt/vol) glucose (M17G) and 5  $\mu\text{g}/\text{ml}$  tetracycline in 100-ml serum bottles without agitation at 30°C. The initial pH of the medium was adjusted to 7.0 by the addition of HCl or NaOH. Growth was followed by measuring the turbidity at 660 nm. In the middle of exponential phase, cells were harvested by spinning them for 10 min at 3,000 rpm when the culture reached a turbidity of 0.6. The pellets were washed two times with 50 mM potassium phosphate (pH 5.8) buffer and, finally, were resuspended in the same buffer.

**Citrate consumption by resting cells.** The assay was performed with a total volume of 1.5 ml with resting cells at an optical density at 660 nm ( $\text{OD}_{660}$ ) of 1.5. Resting cells at an  $\text{OD}_{660}$  of 6 in 50 mM potassium phosphate (pH 5.8) buffer were incubated at 30°C without agitation for 10 min. At time zero, citrate was added at a concentration of 2 mM together with glucose, L-lactate, pyruvate, 2-hydroxyisobutyrate (2-HIB),  $\alpha$ -acetolactate, or acetate at the indicated concentrations.  $\alpha$ -Acetolactate was prepared freshly by saponification of ethyl 2-acetoxy-2-methylacetoacetate (diester of  $\alpha$ -acetolactate) according to reference 13. Ten microliters of ethyl 2-acetoxy-2-methylacetoacetate was added to 1 ml of 180 mM NaOH at 10°C for 20 min in a stirred closed tube. Then, 2 ml of 100 mM maleate (pH 6.0) buffer and 0.35 ml of 100 mM maleic acid or 2.35 ml potassium phosphate (pH 5.8) buffer were added. The final solution was 15.4 mM  $\alpha$ -acetolactate containing equimolar concentrations of acetate and ethanol in maleate (pH 6.0) buffer or potassium phosphate (pH 5.8) buffer. Samples of 100  $\mu\text{l}$  of the cell suspensions were taken every 5 min and immediately centrifuged for 0.5 min at maximum speed in a tabletop centrifuge. The supernatant was stored on ice until further analysis by the enzymatic assays and/or high-performance liquid chromatography (HPLC) (see below). Measurements of the concentrations of citrate, oxaloacetate, and pyruvate were in good agreement between the two methods.

**Enzymatic assays.** Citrate, oxaloacetate, and pyruvate were measured using the commercially available enzymes citrate lyase, L-malate dehydrogenase, and L-lactate dehydrogenase. The assay was performed with microtiter plates. An aliquot of 30  $\mu\text{l}$  of the supernatant was added to 50 mM glycine-glycine (pH 7.8) buffer, giving a total volume of 200  $\mu\text{l}$ , containing 0.45 mM NADH and 0.95 U of L-lactate dehydrogenase. Pyruvate in the sample is converted to L-lactate at the expense of NADH. Oxaloacetate in the same sample was measured after addition of 1  $\mu\text{l}$  (1.9 U) of L-malate dehydrogenase, resulting in the conversion of oxaloacetate to L-malate at the expense of NADH. Subsequent addition of 1  $\mu\text{l}$  (0.032 U) of citrate lyase converts citrate in the sample to oxaloacetate (and pyruvate), resulting in an additional decrease in the NADH concentration equivalent to the citrate concentration present in the samples. The decrease in NADH concentration was measured spectroscopically at 340 nm.

**Colorimetric determination of acetoin and diacetyl.** Acetoin and diacetyl were measured according to reference 43. Acetoin and diacetyl react with guanido groups of creatine in alkaline medium to give a pink color. A volume of 30  $\mu\text{l}$  of the supernatant was mixed with 10  $\mu\text{l}$  of 0.5% (wt/vol) creatine, 10  $\mu\text{l}$  of 5% (wt/vol)  $\alpha$ -naphthol freshly dissolved in 2.5 M NaOH, and 150  $\mu\text{l}$  of water. Color development was measured at  $\text{OD}_{530}$  after 5 min and after 40 min of incubation in the dark at room temperature to determine the amounts of diacetyl and acetoin, respectively. The amount of acetoin was calculated from a calibration curve of known concentrations. Only a trace amount of diacetyl was found in the supernatants, indicating negligible spontaneous nonenzymatic oxidation of  $\alpha$ -acetolactate to diacetyl.

**HPLC analysis.** An aliquot of 10  $\mu\text{l}$  of the supernatants was loaded on an Aminex HPLC-87H anion-exchange column (Bio-Rad Laboratories, Inc., Richmond, CA) operated at 30°C in isocratic mode, using 0.005 M  $\text{H}_2\text{SO}_4$  as the mobile phase and a flow rate of 0.8 ml/min. The samples were analyzed for the presence of 2,3-butanediol, acetate, acetoin,  $\alpha$ -acetolactate, citrate, diacetyl, ethanol, formate, isocitrate,  $\alpha$ -ketoglutarate, L-lactate, oxaloacetate, and pyruvate.  $\alpha$ -Acetolactate was prepared freshly as described above. Concentrations were estimated from standard curves prepared separately for all these compounds and reported as averages from three independent measurements with standard deviations.

**Measurement of internal pH ( $\Delta\text{pH}$ ) and membrane potential ( $\Delta\Psi$ ).** Cells were prepared as described above. Resting cells resuspended to high density (typically containing 50 mg/ml of protein) in 50 mM potassium phosphate (pH 5.8) buffer were loaded with BCECF by mixing 20  $\mu\text{l}$  of the cell suspension with

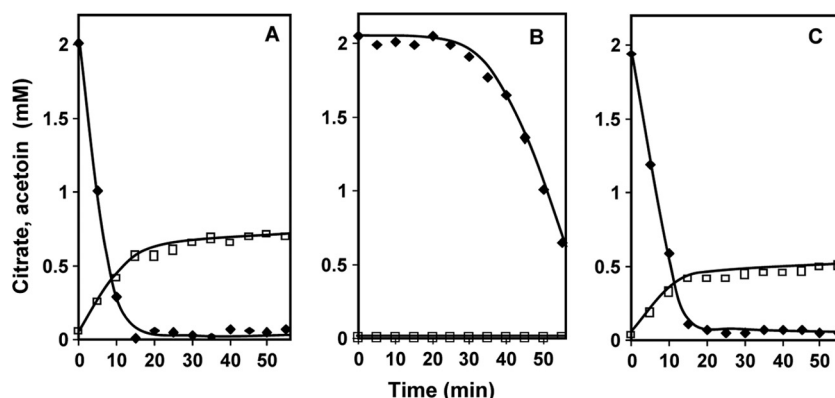


FIG. 2. Citrate metabolism in *L. lactis* IL1403(pFL3). A concentration of 2 mM citrate was added to resting cells in the presence of 0.1 mM glucose (A), no further additions (B), and 0.2 mM L-lactate (C). The citrate (◆) and acetoin (□) concentrations in the supernatant were measured at the indicated time points.

1  $\mu$ l of a 10 mM BCECF solution and 0.5  $\mu$ l of 0.5 M HCl. The suspension was incubated at room temperature for exactly 5 min, after which 1 ml of 50 mM potassium phosphate (pH 5.8) buffer was added. The cells were washed 4 times, resuspended in 200  $\mu$ l of buffer, and kept on ice until use. Fluorescence measurements were performed with a 1-cm cuvette containing 3 ml 50 mM potassium phosphate (pH 5.8) buffer equilibrated at 30°C and 5 to 10  $\mu$ l of BCECF-loaded cells, depending upon the concentration of BCECF in the cells. The cuvette was stirred with a magnetic stirring bar. The excitation and emission wavelengths were 502 and 525 nm, with slit widths of 4 and 16 nm, respectively. The fluorescence signal was sampled every second. The opening of the measurement compartment caused loss of data during the first 5 to 6 s after an addition of substrate to the cuvette. The cytoplasmic pH was calculated as described previously (29).

The membrane potential was measured qualitatively with the fluorescent probe DiSC<sub>3</sub> (37). Decrease in fluorescence intensity correlates with an increase in electrical potential across the membrane. A volume of 5 to 10  $\mu$ l of the cells, resuspended to an OD<sub>660</sub> of 6 in 50 mM potassium phosphate (pH 5.8) buffer, was added to 3 ml of the same buffer in a 1-cm cuvette. DiSC<sub>3</sub> was added from a stock solution in absolute ethanol to give a final concentration of 2  $\mu$ M, and the system was left to equilibrate for 5 min at 30°C. Adding the same volume of absolute ethanol did not affect the citrate consumption rate of the cells. Fluorescence measurements were performed using excitation and emission wavelengths of 500 and 705 nm, respectively, and slit widths of 8 nm.

## RESULTS

**Citrate metabolism by *L. lactis* IL1403(pFL3).** Citrate consumption by resting cells of *L. lactis* IL1403(pFL3) was studied in the presence and absence of glucose. Strain IL1403 encodes all citrate metabolic enzymes on the chromosome except for the citrate transporter CitP (8). CitP is expressed constitutively from the plasmid pFL3 (24). The strain was grown in M17 broth medium supplemented with glucose until the mid-exponential growth phase (OD<sub>660</sub> = 0.6). In the presence of glucose, resting cells resuspended to an OD<sub>660</sub> of 1.5 in 50 mM potassium phosphate (pH 5.8) buffer rapidly consumed citrate, and at the same time, acetoin was produced (Fig. 2A). Diacetyl was not detected as a product in any significant quantity. Glucose at a concentration of 0.1 mM was converted to 0.2 mM L-lactate at the 5-min time point, after which the L-lactate concentration remained constant during the remainder of the experiment. At a 20-fold-higher concentration (2 mM), citrate was consumed at a rate of 0.18 mM/min, which was constant down to citrate concentrations of about 0.2 mM, indicating a high affinity of the pathway for citrate (zero-order kinetics). Acetoin production followed citrate consumption in time at a

rate of 0.05 mM/min. When all citrate was consumed, 0.67 mM acetoin was produced, giving a yield of almost 70% when the fact that the formation of one molecule of acetoin requires two molecules of citrate was taken into account. Acetoin was exclusively produced when citrate was present; no acetoin was produced from glucose in the absence of citrate (not shown). Following full conversion of citrate, the concentration of acetoin slowly increased, suggesting accumulation of a precursor of acetoin during citrate metabolism (Fig. 2A).

In the absence of glucose, citrate metabolism by the same resting cells of *L. lactis* IL1403(pFL3) showed two phases (Fig. 2B). A first phase, during which no significant consumption of citrate was observed, was followed by a phase in which citrate was consumed at a rate of 0.08 mM/min, which is about half the rate observed in the presence of glucose. Remarkably, no acetoin was produced. These results will be discussed in Discussion.

### Exchange and unidirectional transport catalyzed by CitP.

The citrate transporter CitP catalyzes both unidirectional transport of citrate in symport with a proton and bidirectional transport of citrate in exchange with the metabolic end product L-lactate (Fig. 1B). Exchange is catalyzed much more efficiently than symport (4, 27). The properties of CitP suggest that in the presence of glucose, citrate is taken up rapidly in exchange with L-lactate produced by glycolysis and that the first phase observed in the absence of glucose represents slow unidirectional citrate uptake (Fig. 2). Subsequently, the second fast phase would represent an exchange mode in which citrate is taken up in exchange with a metabolic product formed from citrate.

Citrate metabolism by resting cells in the absence of glucose, but in the presence of L-lactate added to the buffer, resulted in a fast consumption of citrate (Fig. 2C). At a concentration of 0.2 mM L-lactate, the rate was 0.17 mM/min, similar to that observed in the presence of 0.1 mM glucose (Fig. 2A). The yield of acetoin produced from citrate in the presence of L-lactate was a bit lower than the yield produced when glucose was present (0.5 and 0.67 mM, respectively). Again, no significant amounts of diacetyl were formed. Similar to what was observed in the presence of glucose, the concentration of acetoin increased slowly following full conversion of citrate (Fig. 2C).



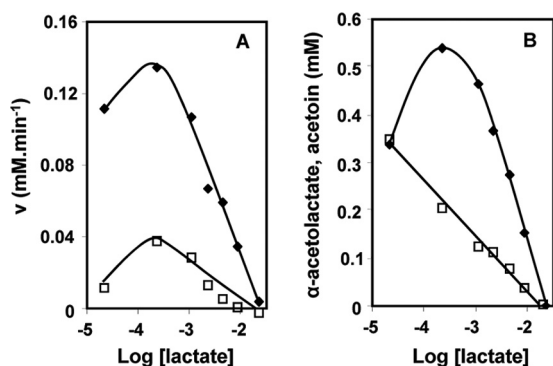


FIG. 3. Effect of L-lactate concentration on citrate metabolism in *L. lactis* IL1403(pFL3). A concentration of 2 mM citrate was added to resting cells in the presence of L-lactate at concentrations ranging from 0.02 mM to 20 mM. (A) Rates of citrate consumption ( $\blacklozenge$ ) and acetoin production ( $\square$ ). Rates were deduced from the decrease in citrate and increase in acetoin concentrations in the period during which 75% of citrate was consumed, assuming zero-order kinetics (Fig. 2C).  $v$ , rate. (B) Concentrations of acetoin ( $\blacklozenge$ ) and  $\alpha$ -acetolactate ( $\square$ ) produced when all citrate was consumed.

A 10-fold-lower concentration of L-lactate added to the buffer or produced from glucose supported rapid metabolism of 2 mM citrate (Fig. 2A and C), which is explained by the “lactate shuttle” depicted in Fig. 1C. L-Lactate exported from the cells by CitP reenters as L-lactic acid by passive diffusion (27). The concentration of L-lactate required to catalyze citrate uptake in the fast citrate/L-lactate exchange mode was remarkably low (Fig. 3A). The rate was optimal at a concentration of 0.2 mM, and still, 80% of this rate was observed at a concentration as low as 20  $\mu\text{M}$ . Higher concentrations of L-lactate were inhibitory, resulting in complete inhibition at a concentration of 20 mM. The acetoin production rate showed the same profile.

The citrate metabolic pathway in LAB is proton motive force (PMF) generating in both the symport and the exchange modes of the transporter (26, 27, 28). The two components of the PMF, pH gradient ( $\Delta\text{pH}$ ) and membrane potential ( $\Delta\psi$ ), generated under the experimental conditions described above, were measured with the fluorescent dye BCECF (29) and the potentiometric probe, 3,3'-dipropylthiocarbocyanine iodide ( $\text{DiSC}_3$ ) (37), respectively. The cytoplasmic pH of the resting cells was very close to the pH of the buffer (Fig. 4). In the presence of 0.1 mM glucose, a significant PMF was generated, presumably the result of proton pumping by  $\text{F}_0\text{F}_1$ -ATPase driven by hydrolysis of ATP produced in glycolysis. The PMF largely consisted of a pH gradient of 1.3 units (Fig. 4A). Upon addition of 2 mM citrate, the internal pH did not increase further but additional PMF was generated as membrane potential (Fig. 4A and D). Addition of citrate to the cells alone resulted in a minor generation of a pH gradient without a change in the membrane potential (Fig. 4B and E). Apparently, the flux through the pathway during the slow phase of citrate consumption (Fig. 2A) was too low to support a significant proton motive force. In contrast, the high flux observed in the presence of 0.2 mM L-lactate resulted in significant increases in both  $\Delta\text{pH}$  (0.6 pH units) and  $\Delta\psi$  (Fig. 4C and F). Importantly, both the BCECF and the  $\text{DiSC}_3$  probes had an inhibitory effect on the rate of citrate consumption (not

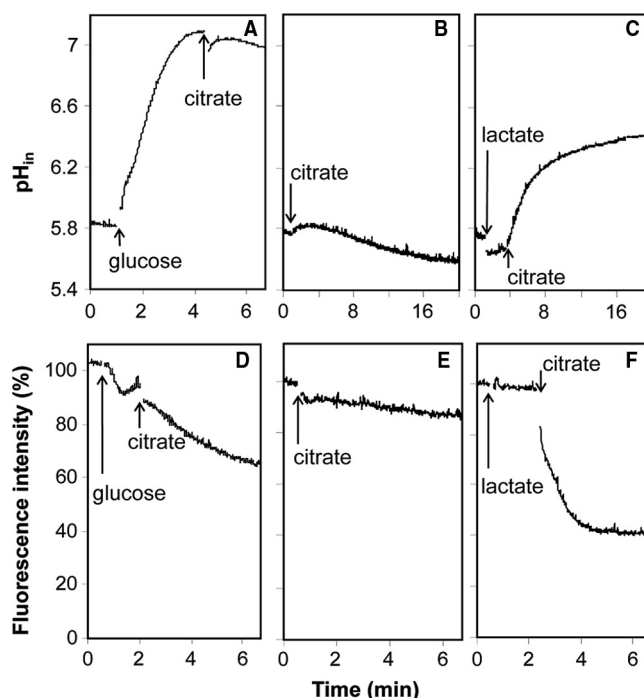


FIG. 4. Energetics of citrate metabolism in *L. lactis* IL1403(pFL3). The internal pH (A, B, C) and membrane potential (D, E, F) of the cells were continuously monitored in time. At the times indicated by the arrows, 0.5 mM glucose and 2 mM citrate (A, D), 2 mM citrate (B, E), and 2 mM citrate and 1 mM L-lactate (C, F) were added. Cells were loaded with BCECF as described in Materials and Methods. Changes in the membrane potential were qualitatively evaluated from the quenching of the potentiometric probe  $\text{DiSC}_3$ .

shown). In the absence of glucose or lactate, the second fast phase (Fig. 2B) was not apparent within 60 min and the energetics of the second phase could not be evaluated. Furthermore, the highest gradients obtained in the presence of L-lactate required a 10-fold-higher concentration than that required for the optimal rate of citrate consumption in the absence of the probes (Fig. 3A).

**Products of citrate metabolism.** Citrate conversion by lactic acid bacteria to acetoin and acetate is redox neutral, and no ATP is produced or consumed (Fig. 1A), indicating that in resting cells, the pathway is driven by the free energy gradient over the overall reaction and counteracted only by the buildup of PMF. Nevertheless, the yield of the conversion was far from 100%, indicating that other products formed preferably, especially when only citrate was present. Table 1 presents the products of citrate metabolism by resting cells of *L. lactis* IL1403(pFL3) for the three experimental conditions discussed above. In the presence of glucose, when the highest yield of acetoin was obtained (67%), two other products were found outside the cells, the precursor of acetoin  $\alpha$ -acetolactate (22%) and a small amount of acetate (11%). Please note that this amount of acetate is in addition to the 2 mM formed in the citrate lyase reaction. Glucose was stoichiometrically converted to two molecules of L-lactate. In the presence of 0.2 mM L-lactate, the lower yield of acetoin (50%) was mainly compensated for by an increased yield of  $\alpha$ -acetolactate (36%), while the additional acetate produced remained more or less

TABLE 1. Rates of citrate consumption and products formed by resting cells of *L. lactis* IL1403(pFL3) from 2 mM citrate in the presence and absence of different substrates

Condition <sup>a</sup>	Concn (mM)	$v^b$ (mM/min)	Product (mM)					Total (mM) <sup>d</sup>
			Oxaloacetate	Pyruvate	$\alpha$ -Acetolactate	Acetate <sup>c</sup>	Acetoin	
Glucose	0.1 <sup>e</sup>	0.18	0	0	0.23 $\pm$ 0.03	0.23 $\pm$ 0.02	0.67 $\pm$ 0.05	2.03 $\pm$ 0.18
L-Lactate	0.2	0.17	0	0	0.36 $\pm$ 0.01	0.25 $\pm$ 0.02	0.50 $\pm$ 0.01	1.97 $\pm$ 0.06
2-HIB	2	0.17	0	0	0.47 $\pm$ 0.08	0.40 $\pm$ 0.03	0.32 $\pm$ 0.01	1.91 $\pm$ 0.12
No addition		0.08 <sup>f</sup>	0.13 $\pm$ 0.01	0.37 $\pm$ 0.03	0.38 $\pm$ 0.06	0.67 $\pm$ 0.10	0	1.86 $\pm$ 0.26
Acetate	2	0.046	0.05 $\pm$ 0.01	0.4 $\pm$ 0.01	0.39 $\pm$ 0.02	0.75 $\pm$ 0.06	0	1.98 $\pm$ 0.10
Pyruvate	2	0.087	0.08 $\pm$ 0.01	0	0.28 $\pm$ 0.08	1.4 $\pm$ 0.04	0	2.04 $\pm$ 0.13
$\alpha$ -Acetolactate/acetate	2/2	0.06	0.09 $\pm$ 0.01	0.75 $\pm$ 0.08	0	1.2 $\pm$ 0.09	0	2.04 $\pm$ 0.18

<sup>a</sup> Substrate present in addition to 2 mM citrate.<sup>b</sup> Cell OD<sub>660</sub> = 1.5. *v*, rate.<sup>c</sup> In addition to acetate formed by citrate lyase.<sup>d</sup> In equivalents of citrate (mM).<sup>e</sup> Converted to 0.2 mM L-lactate.<sup>f</sup> Second phase (Fig. 2).

the same (12%). In the absence of any further additions, when no citrate was converted to acetoin, the product profile changed dramatically. Approximately equal fractions of the flux were directed to  $\alpha$ -acetolactate and acetate (38 and 33%, respectively), while in addition a significant amount of pyruvate (19%) was produced. A minor amount of citrate was converted to oxaloacetate (6%). It follows that in addition to a significant amount of acetate, intermediates of the citrate metabolic pathway to acetoin show up as products outside the cells in amounts that depended on the conditions. In all cases, the increase in time followed the decrease of the citrate concentration in time (not shown). Following full conversion of citrate, the slow increase in the acetoin concentration mentioned above in the presence of glucose and L-lactate (Fig. 2A and C) was accompanied by a slow decrease of  $\alpha$ -acetolactate, indicating the reuptake of the latter by the cells and, subsequently, conversion into the former (not shown). Under all three conditions, an amount of acetate was formed, ranging from ~10 to 40%, indicating that citrate metabolism in resting cells of IL1403(pFL3) diverts to two pathways, yielding acetoin and acetate.

**Exchange with metabolic intermediates.** Excretion of pyruvate,  $\alpha$ -acetolactate, and acetate during the fast second phase of citrate consumption by resting cells when only citrate was present (Table 1 and Fig. 2B) suggests that citrate was taken up in exchange with one or more of these metabolic products. The enhancement of the rate of citrate metabolism by L-lactate provides an assay for substrates of CitP (Fig. 2C). Any weak acid that is a substrate of CitP and membrane permeable should be able to induce rapid citrate consumption by *L. lactis* IL1403(pFL3) cells by the shuttle mechanism depicted for L-lactate in Fig. 1C. In agreement, the fast mode of citrate consumption was observed in the presence of the nonphysiological substrate 2-hydroxyisobutyrate (2-HIB) (22) (Fig. 5A). A concentration of 2 mM 2-HIB was required to get the same rate as that obtained in the presence of 0.2 mM L-lactate. The amount of acetoin produced under this condition was considerably smaller (0.32 mM versus 0.5 mM), which was mainly compensated for by an increase in the production of  $\alpha$ -acetolactate (Table 1). The energetics of the pathways in the presence of 2-HIB and L-lactate were the same, as evidenced by the generation of  $\Delta$ pH and  $\Delta$  $\Psi$ , even though the magnitude of the

latter was lower than that observed in the presence of L-lactate (Fig. 5B and C).

The ability of the weak acids pyruvate,  $\alpha$ -acetolactate, and acetate to function in citrate exchange catalyzed by CitP was measured by their ability to enhance citrate metabolism. At a concentration of 2 mM, pyruvate and acetate resulted in fast consumption of citrate by the resting cells of *L. lactis* IL1403(pFL3) (Fig. 6A). For both substrates, a short delay was observed before the onset of the fast phase. The rates were lower than those observed in the presence of glucose, L-lactate,

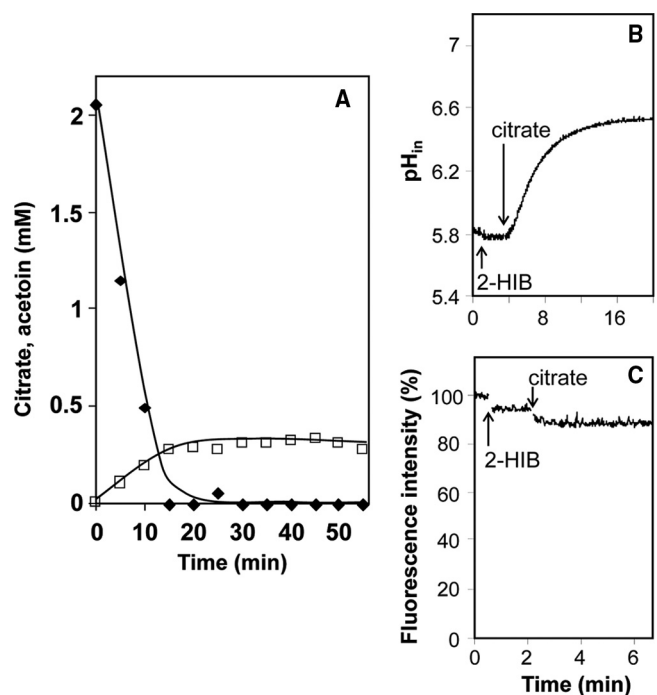


FIG. 5. Citrate metabolism in *L. lactis* IL1403(pFL3) in the presence of 2-hydroxyisobutyrate (2-HIB). (A) Concentrations of 2 mM citrate and 2 mM 2-HIB were added to resting cells, and the concentrations of citrate ( $\blacklozenge$ ) and acetoin ( $\square$ ) in the supernatants were measured. (B) The internal pH (B) and membrane potential (C) of the cells were monitored in time. Concentrations of 8 mM 2-HIB and 2 mM citrate were added to the cells, as indicated by the arrows.

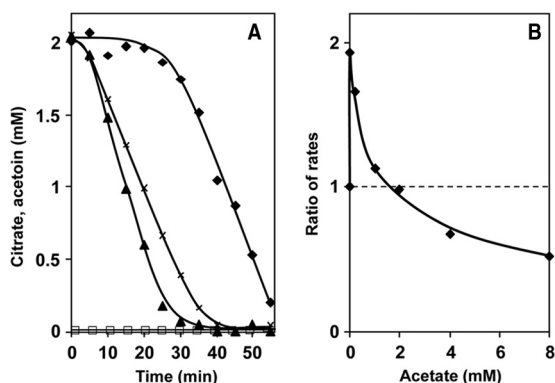


FIG. 6. Citrate uptake in exchange with metabolic intermediates in *L. lactis* IL1403(pFL3). (A) A concentration of 2 mM citrate was added to resting cells in the presence of 2 mM pyruvate ( $\blacktriangle$ ), 2 mM acetate ( $\times$ ), and no further additions ( $\blacklozenge$ ). The citrate ( $\blacktriangle$ ,  $\times$ ,  $\blacklozenge$ ) and acetoin ( $\square$ ) concentrations in the supernatant were measured at the indicated time points. (B) Ratio of the rates of citrate consumption obtained when 2 mM citrate was added to the cells in the presence of  $\alpha$ -acetolactate freshly prepared from the diester (see Materials and Methods) and acetate in the concentration range of 20  $\mu$ M to 8 mM the latter. Freshly prepared  $\alpha$ -acetolactate contains an equivalent concentration of acetate. Rates were deduced from the decreases in citrate concentrations during the second phase, assuming zero-order kinetics.

or 2-HIB but in the same order of magnitude as that observed in the fast second phase in the presence of citrate alone (Table 1). The same experiment with  $\alpha$ -acetolactate was complicated by the fact that the compound is not stable and is commercially available only as a diester. The release of  $\alpha$ -acetolactate from the diester by hydrolysis results in equivalent amounts of acetate and ethanol (see Materials and Methods). The presence of 2 mM ethanol did not have a significant effect on the citrate consumption rate (not shown). At concentrations lower than 2 mM, the equimolar mixture of  $\alpha$ -acetolactate and acetate (and ethanol) induced a higher rate of citrate consumption than that observed in the presence of acetate alone, demonstrating exchange of citrate and  $\alpha$ -acetolactate (Fig. 6B). At higher concentrations,  $\alpha$ -acetolactate in the mixture inhibited the rate observed with acetate alone, the same phenomenon as that observed for L-lactate (Fig. 3A).

The results suggest that uptake of citrate by CitP competes for exchange with the intermediates pyruvate and  $\alpha$ -acetolactate and the end products L-lactate and acetate. In agreement, increasing the concentration of L-lactate added to the cells in the presence of citrate resulted in a decrease of the appearance of  $\alpha$ -acetolactate outside the cells (Fig. 3B). Apparently, more citrate was taken up in exchange with L-lactate added to the cells and less with  $\alpha$ -acetolactate produced by the cells, which, consequently, resulted in a higher production of acetoin. When L-lactate becomes inhibiting, the yield of acetoin goes down again.

In line with the lack of acetoin production in the presence of citrate alone, neither acetate nor pyruvate or  $\alpha$ -acetolactate induced the production of acetoin (Table 1). The product profile in the presence of acetate was very similar to the profile observed with citrate alone; the flux was evenly distributed over the acetoin and acetate pathways, and a significant amount of pyruvate was excreted from the cells. Under these

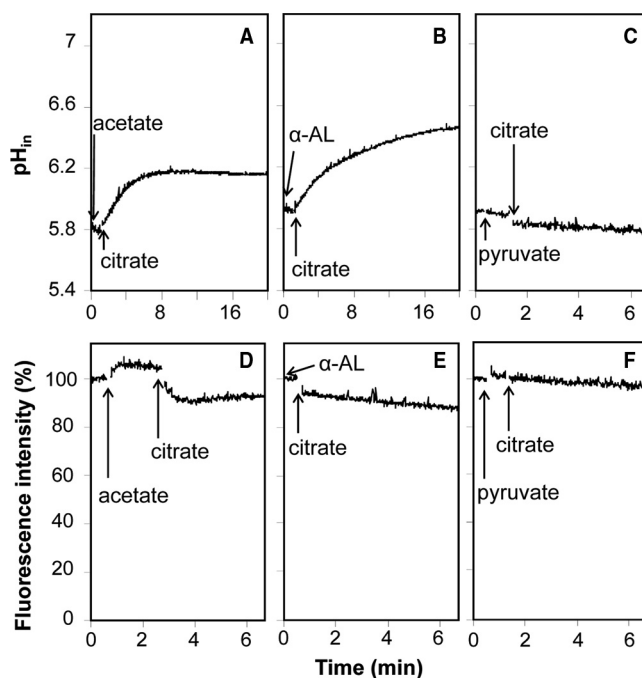


FIG. 7. Energetics of citrate metabolism in *L. lactis* IL1403(pFL3) in the presence of metabolic intermediates. The internal pH (A, B, C) and membrane potential (D, E, F) of the cells were continuously monitored in time. At the time points indicated by the arrows, 2 mM acetate and 2 mM citrate (A, D), 2 mM  $\alpha$ -acetolactate (plus acetate) and 2 mM citrate (B, E), and 8 mM pyruvate and 2 mM citrate (C, F) were added. Cells were loaded with BCECF as described in Materials and Methods. Changes in the membrane potential were qualitatively evaluated from the quenching of the potentiometric probe DiSC<sub>3</sub>.

conditions, a pH gradient of 0.4 units was generated and, in addition, membrane potential was generated (Fig. 7A and D). In the presence of pyruvate and  $\alpha$ -acetolactate (plus acetate), the product profile changed considerably. Both prevented the production of additional amounts of the added compound and shifted the flux to acetate production. In the presence of 2 mM  $\alpha$ -acetolactate plus 2 mM acetate, the pathway to  $\alpha$ -acetolactate and acetoin was completely absent and only pyruvate and acetate were produced. Then, a pH gradient of 0.5 units was generated without an effect on the membrane potential (Fig. 7B and E). Remarkably, citrate metabolism in the presence of pyruvate did not result in the generation of a detectable proton motive force (Fig. 7C and F).

## DISCUSSION

**Metabolic state of the cells.** In this study, citrate metabolism was studied in the presence and absence of other substrates in resting cells of *L. lactis* IL1403(pFL3). The cells were grown in batch culture in rich medium (M17) at an initial pH of 7 and supplemented with glucose as an energy and carbon source. The cells were harvested at a relatively low density ( $OD_{600}$  of 0.6) when the pH had dropped to 6.8. Under these conditions, *L. lactis* ferments glucose by a homofermentative metabolism, producing L-lactate (14, 42). In the experiments, citrate metabolism is catalyzed by the complement of enzymes present in the cells under those growth conditions. The metabolic state is

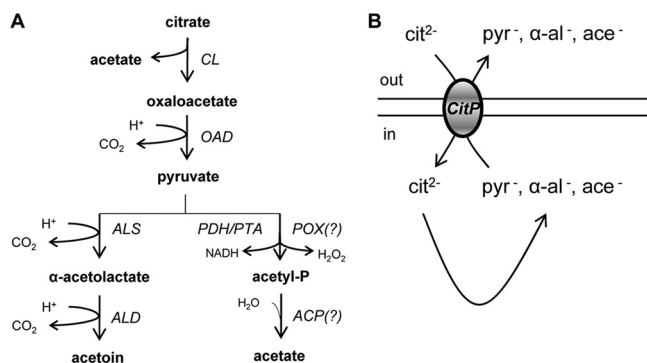


FIG. 8. Citrate metabolism in *L. lactis* IL1403(pFL3). (A) Pathway for the conversion of citrate to acetoin and to acetate. Enzymes: CL, citrate lyase; OAD, oxaloacetate decarboxylase; ALS, α-acetolactate synthase; ALD, α-acetolactate decarboxylase; POX, pyruvate oxidase; PDH, pyruvate dehydrogenase complex; PTA, phosphotransacetylase; ACP, acylphosphate phosphohydrolase. The stoichiometry of the reactions was not taken into account. (B) Exchange of divalent citrate [ $\text{cit}^{2-}$ ] and citrate metabolic products (pyruvate [ $\text{pyr}^-$ ], α-acetolactate [ $\alpha\text{-al}^-$ ], and acetate [ $\text{ace}^-$ ]) catalyzed by CitP.

typical for exponential growth when energy is not limiting and not typical for citrate fermentation conditions.

In citrate-fermenting *L. lactis* strains, the citrate transporter CitP is encoded on an endogenous plasmid (24), while the metabolic enzymes are encoded on the chromosome (8). Expression of the *citP* gene is induced by the presence of citrate under acidic conditions in line with the physiological function of the citrate fermentation pathway in acid stress resistance in *L. lactis* CRL264 (15, 24). Strain IL1403 (10) is a plasmid-free strain and, therefore, a citrate permease mutant of the biovar diacetylactis. Transfer of plasmid pFL3 to IL1403, yielding IL1403(pFL3), converted to the strain the ability to produce acetoin and diacetyl (9). Plasmid pFL3 contains the lactococcal *citP* gene under the control of the *S. pneumoniae* *polA* promoter (24). Expression of neither the transporter nor the plasmid copy number is controlled by citrate or the pH of the medium. In agreement, resting cells of *L. lactis* IL1403(pFL3) grown in the medium with an initial pH of 6 or 7 or in the presence or absence of 10 mM citrate showed no significant difference in citrate consumption rate (not shown).

**Broadening of CitP substrate specificity.** The citrate transporter CitP plays a pivotal role in the energetics of the citrate fermentation pathway in LAB by catalyzing electrogenic exchange of divalent citrate and monovalent lactate. It was shown before that CitP has a broad substrate specificity, translocating a wide range of physiological and nonphysiological 2-hydroxycarboxylates (4, 6). The present study demonstrates an even broader specificity of CitP. The intermediates α-acetolactate and pyruvate and the end product acetate were shown to be substrates of CitP (Fig. 8B). α-Acetolactate was not a surprise, because it contains the 2-hydroxycarboxylate motif. Also, some activity with the 2-ketoacid pyruvate was already observed in studies with membrane vesicles (4). Possibly, the hydrated form of pyruvate (2,2-dihydroxypropionate) is the real substrate. Exchange activity with acetate was not observed before. It may be noted that CitW of *K. pneumoniae*, another member of the 2-HCT family, catalyzes citrate/acetate exchange and is believed to function in citrate fermentation in

this Gram-negative bacterium (20). The broad specificity of CitP for physiological substrates has implications for the citrate metabolic pathway in resting cells. A significant flux through the pathway requires CitP to operate in the exchange mode. Depending on their availability and concentration inside and outside the cell, CitP uses pyruvate, α-acetolactate, acetate, or L-lactate in the exchange mode to take up citrate from the medium. In their absence, citrate is taken up by CitP operating in the slow symport mode (5) and an internal pool of intermediates and the product acetate has to build up before CitP can switch to the fast exchange mode, which results in a lag time observed in the consumption of citrate (Fig. 2B). Then, the metabolic intermediates pyruvate and α-acetolactate are produced as the products of the pathway. In their presence, CitP operates in the exchange mode from the beginning via the shuttle mechanism depicted in Fig. 1C. The product profile depends on the competition between the different substrates of CitP present in the system.

**Diverse fates of citrate.** Analysis of the products formed from citrate under various conditions by resting cells of *L. lactis* IL1403(pFL3) revealed the metabolic pathway depicted in Fig. 8A. Citrate is converted to pyruvate, followed by branching of the pathway yielding either acetoin or acetate. The profile of product formation under various conditions can be divided into two main groups (Table 1). In one group, citrate is taken up by CitP in exchange with a substrate that is not formed from citrate, while in the other group the substrate is formed from citrate. L-Lactate, produced from glucose or added to the buffer, and 2-HIB are not part of citrate metabolism, and their involvement is restricted to the transport step. With these substrates, the rate of citrate consumption was highest and 80 to 90% of pyruvate produced was directed to the acetoin route. Still, a significant fraction did not make it to acetoin and was excreted as α-acetolactate, indicating that this 2-hydroxycarboxylate competes successfully with L-lactate and 2-HIB in the exchange reaction catalyzed by CitP. In the other group, the flux distribution is mainly determined by the availability of exchangeable substrates. The overall rate of citrate consumption was lower, and the fraction of pyruvate directed to the acetoin route dropped to 0 to 40%. All α-acetolactate produced was used to take up citrate in the exchange process, and consequently, no acetoin was produced under these conditions. In addition, a significant fraction of pyruvate, up to 40%, was spent to take up citrate and ended up outside the cells. The remainder of the flux was directed to the acetate route, which amounted to 60 to 70% in the presence of the intermediates pyruvate and α-acetolactate.

**Energetics of citrate metabolism.** Citrate metabolism in LAB yielding acetoin results in the generation of PMF by a secondary metabolism that involves membrane potential generation by electrogenic citrate/lactate exchange and pH gradient generation by proton consumption in the decarboxylation reactions in the pathway. The two contributions are indirectly coupled since the specificity of CitP determines the net number of protons that enter the cell in the transport step (Fig. 1). Citrate metabolism in resting cells of *L. lactis* IL1403(pFL3) generated the greatest PMF in the presence of L-lactate and 2-HIB, conditions characterized by the highest flux through the pathway and the highest yield of acetoin (see above). These conditions reflect the situation in growing cells during citrate/



glucose cometabolism. Under conditions where citrate was taken up in exchange with the intermediates pyruvate and  $\alpha$ -acetolactate and the end product acetate, when the flux was lower and the routing more diverse, the generated PMF was less or even absent. The PMF generated will be the sum of the contributions of the fluxes to external pyruvate,  $\alpha$ -acetolactate, and acetate, suggesting that not all routes are equally effective in PMF generation. In particular, in the presence of pyruvate when the highest yield of acetate was observed, no PMF was detected (Fig. 7C), strongly suggesting that the acetate route does not generate metabolic energy.

**Pathway to acetate.** The lack of PMF generation in the pathway from pyruvate to acetate is surprising since normally the conversion proceeds via acetate kinase and yields ATP. Based on the enzyme complement encoded in the genome of *L. lactis* IL1403 (8), three different routes result in the conversion of pyruvate to acetate, all three proceeding through acetyl-P. Acetyl-P may be formed from acetyl-coenzyme A (CoA) produced from pyruvate by pyruvate-formate-lyase (PFL) or pyruvate dehydrogenase (PDH) and directly from pyruvate by pyruvate oxidase (POX). PFL produces formate in addition, which was not detected as a product, ruling out the involvement of PFL, which is known to act only under strict anaerobic conditions (1). PDH produces NADH in addition, which has to be reoxidized to allow turnover through the route (39). *L. lactis* is an aerotolerant lactic acid bacterium that contains NADH oxidases (encoded by the *noxC*, *noxD*, *noxE* genes [8]) that protect the cell during aerobic growth (11). NOX enzymes catalyze oxidation of NADH to  $\text{NAD}^+$  by molecular oxygen and are induced under aerobic conditions (7, 23, 38). The combination of PDH and NOX enzymes could account for the conversion of pyruvate to acetyl-CoA under the conditions of the experiment. Acetyl-CoA produced by PDH is normally converted to acetaldehyde and ethanol, both of which were not detected in the supernatants, which leaves only the conversion to acetyl-P catalyzed by phosphotransacetylase (PTA). The third enzyme, POX (encoded by the *poxL* gene), produces hydrogen peroxide in addition to acetyl-P. Small amounts of  $\text{H}_2\text{O}_2$ , amounting to a small percentage of the acetate produced, were detected in the supernatants (not shown), suggesting that the enzyme does not play an important role, if any. However, an intriguing possibility might be that  $\text{H}_2\text{O}_2$  produced by POX is used to reoxidize NADH produced by PDH. The enzyme catalyzing the reaction is alkyl hydroperoxide reductase (gene name *ahpC*), which plays a role in detoxification (8, 19). Acetyl-P is converted by acetate kinase to acetate. However, the acetate kinase reaction is coupled to the production of ATP, which should result in the generation of PMF. ATP produced from as little as 0.1 mM glucose generated a pH gradient of 1.3 units (Fig. 4A). Citrate metabolism yielding 1.4 mM acetate did not generate PMF (Table 1 and Fig. 7C), strongly suggesting that no ATP is formed and that acetate kinase is not involved. Possibly, the enzyme encoded by the *yffC* gene (8), which is homologous to acylphosphate phosphohydrolases that catalyze hydrolysis of acetyl-P, plays a role. The details of the pathway from pyruvate to acetate identified here still have to be resolved.

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